Title
Pathways for activation of the ras-oncogene-encoded *p21* protein.

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Source
Ann Clin Lab Sci, 22(5):323-42 1992 Sep-Oct

Abstract
The ras-oncogene-encoded *p21* protein is known to cause a large number of human tumors. This protein differs from its normal counterpart protein, which is present in all eukaryotic cells, in that it contains a single amino acid substitution at critical positions in the polypeptide chain, such as at Gly 12, Gly 13, Ala 59, and Gln 61. Using computer-based molecular modeling, it has been found that one region of this protein that is a candidate for interacting with other intracellular proteins is the region from residues 35 to 47. In oocyte microinjection experiments, it was found that this peptide strongly inhibits the mitogenic effects of oncogenic (Val 12-containing) *p21* but does not inhibit the cellular effects of activation of normal *p21* protein. Furthermore, it has been shown that the cellular effects of oncogenic *p21* protein can be completely inhibited by selectively blocking protein kinase C (PKC) with a highly specific inhibitor of this protein, CGP 41 251, a staurosporine derivative. This inhibitor, however, only weakly inhibits the effects of normal cellular ras-*p21* protein. In addition, a photoaffinity-labeled *p21* protein has been microinjected into NIH 3T3 fibroblasts and have isolated intracellular proteins of MW 35, 43 and 61 kda covalently bound to it. The 43 kda protein is the major one and appears to be critical to the functioning of the *p21* protein. Our results suggest that oncogenic and normal *p21* proteins utilize overlapping but distinct pathways; the oncogenic pathway can be blocked selectively and requires the activation of PKC and the presence of the 43 kda protein.

Language
Eng

Unique Identifier
92398292

Major MeSH Headings
Proto-Oncogene Protein *p21* (ras) AI/CH/GE/*PH PROTO ONCOGENE PROTEIN P A 021 RAS

Minor MeSH Headings
Amino Acid Sequence ; Animal ; Microinjections ; Molecular Sequence Data ; Neoplasms GE ; Oocytes PH ; Protein Conformation ; Protein Kinase C AI ; Protein Kinase C ME ; Second Messenger Systems ; Support, Non-U.S. Gov't ; Support, U.S. Gov't, P.H.S. ; Xenopus laevis

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Publication Type
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ISSN
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UNITED STATES

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9212

EXHIBIT
A

☐ 1: NM_008284 **Mus musculus Harvey rat sarcoma virus oncogene (Hras1), mRNA**


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REFERENCE   1  (bases 1 to 570)
  AUTHORS   Przybojewska,B. and Pluciennicak,G.
  TITLE     Nucleotide sequence of c-H-ras-1 gene from B6C3F1 mice
  JOURNAL   Acta Biochim. Pol. 43 (3), 575-578 (1996)
  MEDLINE   97080708
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EXHIBIT
B

☐ 1: Q61411 **TRANSFORMING PROTEIN P21/H-RAS-1 (C-H-RAS)**

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            created: Nov 1, 1997.
            sequence updated: Nov 1, 1997.
            annotation updated: May 30, 2000.
            xrefs: gi: gi: 895862, gi: gi: 895863
            xrefs (non-sequence databases): HSSP P01112, MGD MGI:96224, PFAM
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KEYWORDS    Proto-oncogene; GTP-binding; Prenylation; Palmitate; Lipoprotein.
SOURCE      house mouse.
  ORGANISM  Mus musculus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.
REFERENCE   1  (residues 1 to 189)
  AUTHORS   Przybojewska,B. and Plucienniczak,G.
  TITLE     Nucleotide sequence of c-H-ras-1 gene from B6C3F1 mice
  JOURNAL   Acta Biochim. Pol. 43 (3), 575-578 (1996)
  MEDLINE   97080708
  REMARK    SEQUENCE FROM N.A.
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            the EMBL outstation - the European Bioinformatics Institute.
            The original entry is available from http://www.expasy.ch/sprot
            and http://www.ebi.ac.uk/sprot
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            [ENZYME REGULATION] ALTERNATE BETWEEN AN INACTIVE FORM BOUND TO GDP
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EXHIBIT
C

☐ 1: U09507 **Mus musculus p21 (Waf1) mRNA, complete cds**


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DEFINITION  Mus musculus p21 (Waf1) mRNA, complete cds.
ACCESSION   U09507
VERSION     U09507.1  GI:595302
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REFERENCE   1  (bases 1 to 855)
  AUTHORS   Huppi,K., Siwarski,D., Dosik,J., Michieli,P., Chedid,M., Reed,S.,
            Mock,B., Givol,D. and Mushinski,J.F.
  TITLE     Molecular cloning, sequencing, chromosomal localization and
            expression of mouse p21 (Waf1)
  JOURNAL   Oncogene 9 (10), 3017-3020 (1994)
  MEDLINE   94366751
REFERENCE   2  (bases 1 to 855)
  AUTHORS   Huppi,K.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-MAY-1994) Konrad Huppi, Lab. of Genetics, National
            Cancer Institute, National Institutes of Health, Building 37, Rm.
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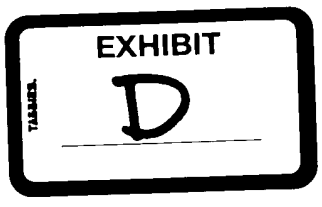

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Recombinant DNA by Watson, Gilman, Witkowski, and Zoller; 2nd edition, 1992 Scientific American Books, New York (8)

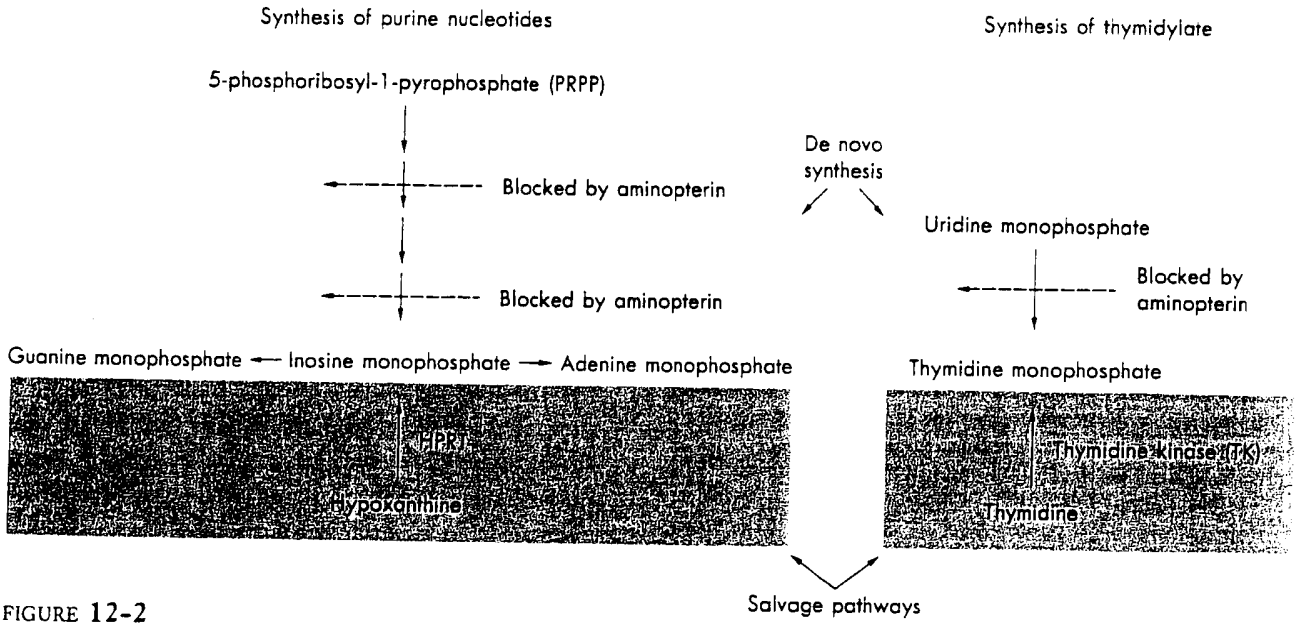

FIGURE 12-2
The metabolic basis for the genetic selection for *tk*. Mammalian cells possess two distinct routes for synthesizing deoxynucleoside triphosphates for DNA synthesis. They can make them from scratch (*de novo* synthesis, shown in blue boxes), or they can salvage free purine and pyrimidine bases from intracellular or extracellular sources (pathways shown in pink boxes). Aminopterin blocks two steps in the biosynthesis of purines and one in the biosynthesis of thymidine. If cells are provided with hypoxanthine and thymidine, they can survive aminopterin treatment by using the salvage pathways. The key salvage enzyme for thymidine nucleotides is thymidine kinase (TK), which phosphorylates free thymidine to thymidine monophosphate. Cells that carry mutations in the gene encoding this enzyme, *tk*, cannot grow in medium containing hypoxanthine, aminopterin, and thymidine (*HAT medium*). Cells that have gained a *tk* gene by transfection can grow in HAT. This selection can also be used for *hprt*, the gene that encodes the key salvage enzyme (hypoxanthine phosphoribosyltransferase—HPRT) for purines.

be tested individually by transfection, allowing the localization of functional genes to specific DNA fragments. Ultimately, when it became possible to clone tumor virus DNA, gene transfer experiments of this type established that cloned DNA, propagated exclusively in bacteria, had precisely the same biological activity as DNA isolated directly from viruses. The significance of these early tumor virus gene transfer experiments for modern biology was profound.

Selectable Markers That Work in Mammalian Cells Allow Gene Transfer by Cotransformation

Stable transfection

One of the basic principles of recombinant DNA technology is the use of biological markers to identify cells carrying recombinant DNA molecules. In bacteria, these are commonly drug-resistance genes. We use drug resistance to select bacteria that have taken up cloned DNA from the much larger population of bacteria that have not. In the early mammalian gene transfer experiments involving viral genes, the transfer of exogenous DNA into cells was detected because the DNA had a biological activity—it led to production of infectious virus or produced stable changes in the growth properties of the transfected cells. But what if we want to study genes whose function we cannot detect simply by looking at the transfected cells? Finding the transfected cells is not a trivial problem, because even the best transfection methods result in stable transfer to only one cell in a thousand.

The solution came from studies of a more complex DNA tumor virus, herpes simplex virus (HSV). HSV was found to contain a gene (*tk*) encoding an enzyme, thymidine kinase, which catalyzes a step in the synthesis of thymidine triphosphate (TTP), one of the four precursor nucleotides for DNA synthesis (Figure

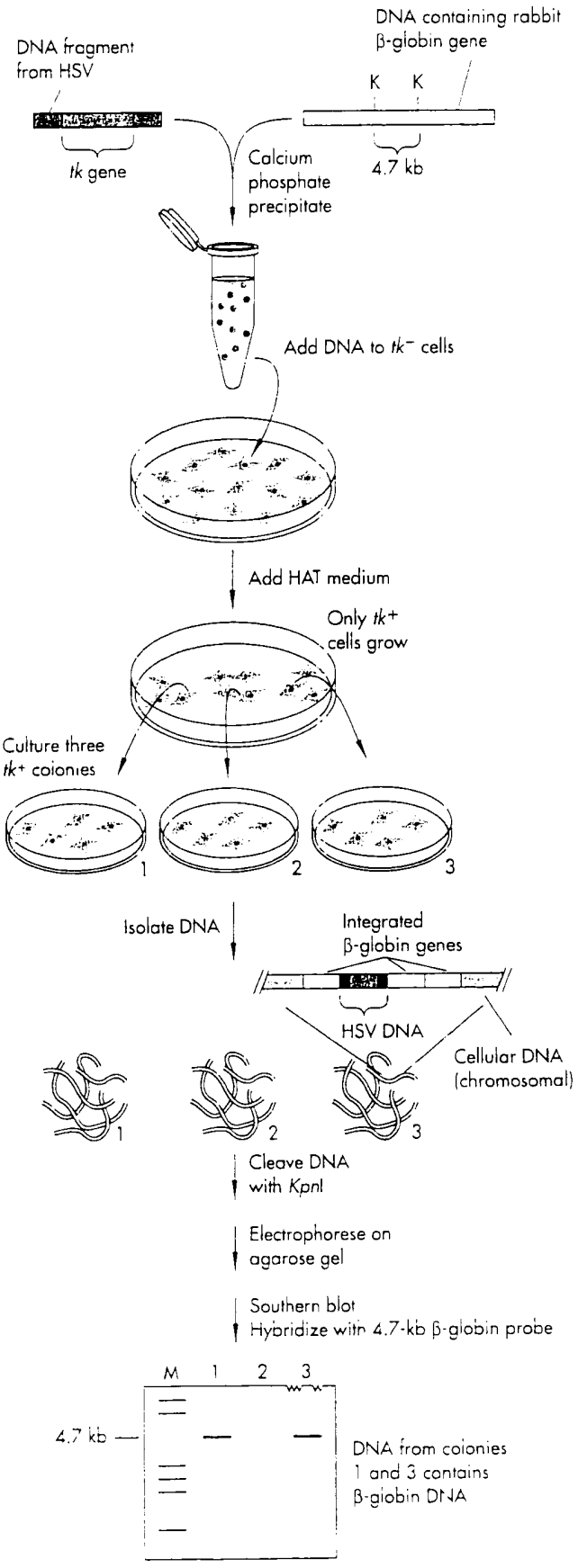

FIGURE 12-3
Transfer of a nonselectable gene by cotransfection with a marker gene. A herpes simplex virus DNA fragment carrying the *tk* gene was mixed with a fragment carrying the rabbit β-globin gene. The two fragments were applied to dishes of mouse tk^- cells as a calcium phosphate precipitate. The precipitate was removed, and the cells were incubated in HAT medium, which supports only the growth of tk^+ cells (see Figure 12-2). After 2 to 3 weeks, colonies of tk^+ cells grew up on the dishes. The colonies were picked and grown up. Chromosomal DNA was isolated, digested with the restriction enzyme *Kpn*I, (which cleaves at the sites indicated by "K" in the β-globin gene), and analyzed by Southern blotting, using a radioactive probe that detected the rabbit β-globin sequence in the fragment generated by cleavage with *Kpn*I. Most of the tk^+ colonies had also acquired the rabbit-β-globin gene. Detailed analysis of the structure of the transfected DNA in these clones showed that it integrated as large arrays carrying multiple copies of both transfected fragments, usually at one or a few chromosomal sites. M, molecular size markers.

12-2). Years earlier, cell lines had been isolated that were deficient in this activity, presumably owing to inactivation of the cellular genes encoding this enzyme. Infection of these tk^- cells with HSV and, in later experiments, transfection with naked HSV DNA were able to correct the defect in thymidine kinase activity in these cells, allowing them to grow in a specially concocted medium in which thymidine kinase is absolutely required for the cells to make DNA (and thus, to multiply). It was quickly realized that the HSV *tk* gene was functioning as a selectable genetic marker in much the same way that drug-resistance genes worked in bacteria, to allow rare transfected cells to grow up out of a much larger population that did not take up any DNA.

Could such a marker be used to identify cells that have taken up a gene for which there is no simple selection? The answer came from experiments like the one depicted in Figure 12-3. Researchers isolated a fragment of HSV DNA carrying the *tk* gene, simply mixed the *tk* DNA with a another DNA fragment carrying a rabbit globin gene, and applied the mixture of DNA fragments to cultures of tk^- cells as a calcium phosphate precipitate. The cells were transferred to *selective* growth medium, which permits growth only of cells that took up a functional *tk* gene, and after a few weeks, the resulting colonies of growing cells were examined by Southern blotting to determine whether the cells had taken up the rabbit globin DNA as well. The vast majority had done so.

These experiments led to the view that while few cells in the culture took up DNA, those that did took up a great deal of it (perhaps 1000 kb or more). And physically unlinked fragments became covalently joined once inside cells, presumably through the action of cellular DNA ligases. The complexes somehow integrated into chromosomal DNA at only one or a few locations. These large complexes of transfected DNA usually contained many copies of each fragment. Thus, cells selected for the presence of one fragment (for example, the *tk* gene) were almost always found also to contain the fragment for which no selection was applied. Moreover, when cells from a single transfected colony were examined, they could be shown to be clonal in the same way a transformed bacterial colony is—all cells in the colony appeared to contain the same arrangement of transfected DNA integrated at the same site in the host cell genome, meaning that they were all descendants of a single transfected cell. Thus, at cell division, the transfected DNA must be faithfully replicated along with the rest of the DNA in the cell. The upshot of all this was that any gene could now be stably transferred into mammalian cells simply by mixing it with a *tk* gene. The cells that took up DNA could be easily selected via the *tk* marker, and surviving colonies could be examined for the presence of the other gene with the expectation that, with high probability, it would be there.

But *tk* is a marker of limited utility, because it can be used only in cells carrying mutations in their own *tk* genes. The ideal marker would permit a simple dominant genetic selection that could operate in any cell the experimentor wished to use. Many such markers have now been developed (Table 12-1). The most commonly used marker in mammalian cells is, ironically, a bacterial drug-resistance gene that confers resistance to a neomycin-related drug, G418, which kills mammalian cells by blocking protein synthesis. The marker gene encodes an enzyme that destroys the drug.

Exogenous DNA Is Transiently Expressed in Many Cells Immediately Following Transfection

Thus far, we have discussed the use of gene transfer to incorporate new DNA *stably* into the genome of the transfected cell. This is a rare event, occurring in one in a thousand to one in a million cells. Many more cells, up to half, actually take up DNA in a transfection but fail to integrate it. In these cells, the DNA persists in the nucleus for several days before disappearing (Figure 12-4). During this period, however, the transfected DNA is subject to many of the regulatory activities that control the expression of endogenous genes in the chromosomes.

Researchers have exploited this window of *transient expression* in several ways. Transient expression is used

TABLE 12-1
Dominant Selectable Markers Used in Transfection Experiments

ENZYME (abbreviation)	DRUG FOR SELECTION	SELECTION MECHANISM
Aminoglycoside phosphotransferase (APH)	G418 (inhibits protein synthesis)	APH inactivates G418
Dihydrofolate reductase (DHFR): Mtx-resistant variant	Methotrexate (Mtx; inhibits DHFR)	Variant DHFR resistant to Mtx
Hygromycin-B-phosphotransferase (HPH)	Hygromycin-B (inhibits protein synthesis)	HPH inactivates hygromycin-B
Thymidine kinase (TK)	Aminopterin (inhibits de novo purine and thymidylate synthesis)	TK synthesizes thymidylate
Xanthine-guanine phosphoribosyltransferase (XGPRT)	Mycophenolic acid (inhibits de novo GMP synthesis)	XGPRT synthesizes GMP from xanthine
Adenosine deaminase (ADA)	9-β-D-xylofuranosyl adenine (Xyl-A; damages DNA)	ADA inactivates Xyl-A

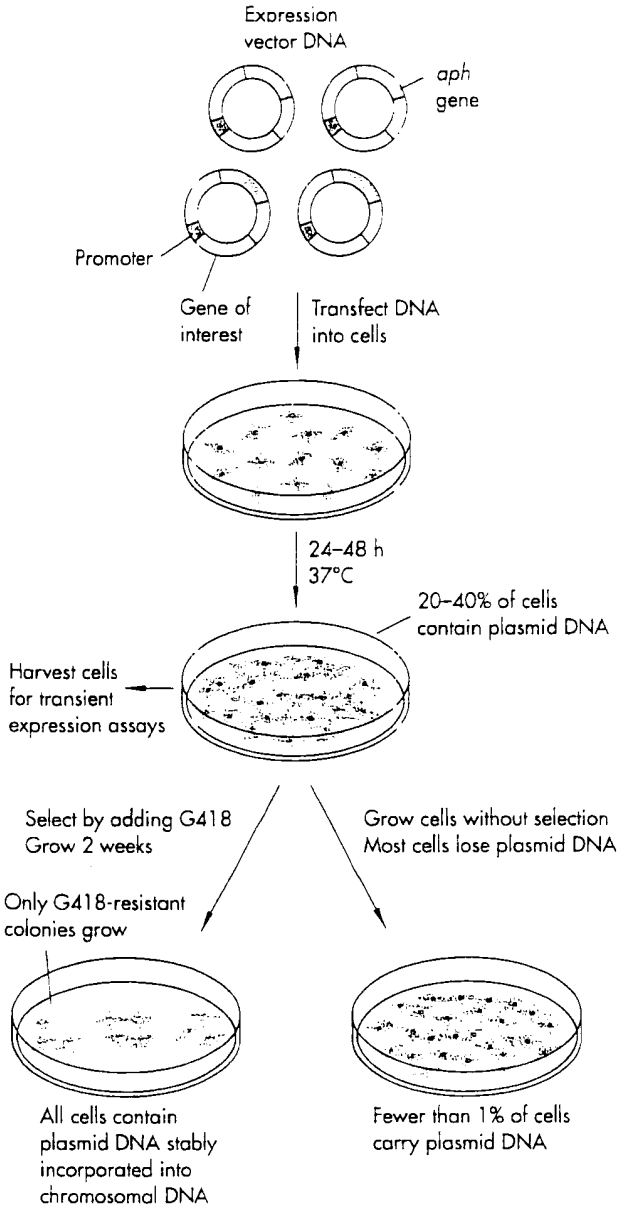

FIGURE 12-4
Transfected DNA is maintained transiently in many cells, but stably integrates in only a few. During the first 48 hours following transfection, as many as 50 percent of the cells in the culture carry the transfected DNA. This is termed the *transient phase*, and for many kinds of experiments, the cultures are used at this point. As the cells continue to incubate, the transfected DNA is progressively lost from most of them (owing to a combination of degradation and dilution, because unintegrated DNA is not usually replicated along with the host cell chromosomes). In only a few cells on the dish does the DNA become stably integrated into a chromosome. Isolating those cells and their descendants requires the application of a *selection* for a transfected marker gene (left). The selection kills off all the cells that failed to integrate transfected DNA, allowing the rare integrants to grow up into large isolated colonies (clones), which can be recovered for further analysis.

as a rapid assay to map the regulatory elements in a gene that control transcription and RNA processing. For example, in the most commonly used assay for studying the activity of mammalian promoters and enhancers, plasmids carrying cloned promoters (often with different sequences deleted or mutated) are transfected into cells, and the cells are harvested 48 to 72 hours later, while the cells are in the transient expression phase. During this period, active promoters are recognized and used by the transcription machinery of the cells, resulting in transcription of downstream sequences. Often these downstream sequences carry a *reporter gene*, such as the bacterial *CAT* gene, that encodes an enzyme that is simple to assay (see Figure 9-2). Or the amount of RNA transcribed from the transfected genes can be measured directly by hybridization to radiolabeled gene probes. Another application of transient expression is the production of large amounts of protein or RNA from a cloned gene. The amount of protein and RNA produced from a transiently expressed transfected gene can be high.

From our studies of viral and cellular genes, we have learned many of the strategies used by these genes to achieve high-level expression. Modern expression vectors are designed on the basis of what we have learned and incorporate strong viral or cellular promoters and efficient translational initiation signals. In addition, most vectors incorporate an intron, since splicing increases the efficiency of export of mRNA from the nucleus for translation. Vectors can be engineered with protein coding sequences that direct the expressed protein to particular locations in the cell, such as secretory vesicles, or that tag the protein with some additional peptide sequence that can be used to purify it. An example of such a vector is shown in Figure 12-5.

Gene Amplification Is Used to Achieve High-Level Protein Expression

Gene amplification is a special application of stable transfection, used to obtain very high levels of expression of a transfected gene. When cell cultures are treated with methotrexate (Mtx), an inhibitor of a critical metabolic enzyme, dihydrofolate reductase (DHFR), most cells die, but eventually some Mtx-resistant cells grow up. Upon examination, the resistant cells are found to have *amplified* their *dhfr* genes; that is, they